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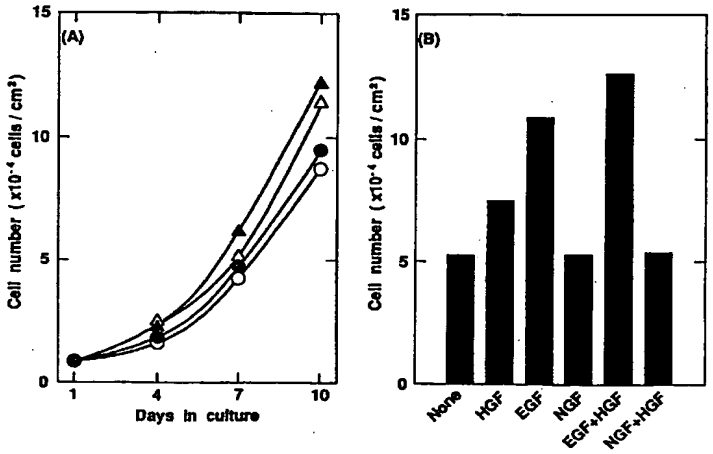
(54) REMEDY FOR CRANIAL NERVE DISORDER

(57) A remedy for cranial nerve disorder containing a hepatocyte growth factor (HGF) as the active ingredient, and a method of treating cranial nerve disorder by administering HGF, which has the effect of maintaining the survival of cranial nerve cells and can achieve regeneration and repair of damaged brain and nerves. Therefore the invention remedy and treatment method are useful for preventing or treating various cranial nerve disorders such as dementia, senile dementia of Alzheimer type, cerebral stroke and cerebral infraction.

Fig. 3



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Description

Technical Field

The present invention relates to a therapeutic agent for disorder in brain and nerve. More particularly, it relates to a therapeutic agent for disorder in brain and nerve containing HGF (hepatocyte growth factor) as an active ingredient.

Background Art

HGF is a protein discovered by the present inventor in the serum of rats with a regenerating liver as a factor inducing proliferation of mature hepatic parenchymal cells in vitro (Biochem. Biophys. Res. Commun., 122, 1450, 1984). The inventor further succeeded in isolating HGF from rat platelets (Proc. Natl. Acad. Sci., 83, 6489, 1986, FEBS Letter, 22, 311, 1987), and determined a part of its amino acid sequences. On the basis of the clarified amino acid sequences of HGF, the inventor carried out cloning of human and rat HGF cDNAs, and succeeded in obtaining the hepatic parenchymal cell growth factor as a recombinant protein using the cDNA in animal cells (human HGF: Nature, 342, 440, 1989; rat HGF: Proc. Natl. Acad. Sci., 87, 3200, 1990).

The molecular weight of HGF is 82 to 85 kD in SDS-polyacrylamide gel electrophoresis. The rat HGF molecule has a heterodimer structure of α-chain composed of 463 amino acid residues and β-chain composed of 233 amino acid residues, and both α-chain and β-chain are crosslinked to each other with one disulfide bond and have two glucosamine-type sugar chain binding sites. The human HGF also possesses almost the same physiological activity, and has α-chain composed of 463 amino acid residues and β-chain composed of 234 amino acid residues. The α-chain has 4 kringle structures similar to that of fibrinolytic enzyme plasmin, and the amino acid sequence of β-chain has about 37% homology with β-chain of plasmin having serine protease activity. The homology of amino acid sequence between rat HGF and human HGF is very high, i.e. 91.6% in α-chain and 88.9% in β-chain, and their activities can be utterly exchangeable.

HGF discovered as a factor for specifically proliferating the hepatic parenchymal cells has been disclosed to show various activities in the body as a result of recent studies by the inventor and other researchers, and it is expected to be applied in remedies for humans and animals, as well as the subject of study.

The inventor proved that HGF acts as a growth factor not only on hepatocytes but also widely on epithelial cells, and have completed several inventions. In Japanese Patent Kokai No. 49246/1992, the inventor described the development and application of HGF as a medicine for renal diseases on the basis of the action of HGF to promote cell proliferation in proximal tubule of the kidney. On the basis of the HGF action to promote proliferation of normal epithelial cells such as melanocytes and keratinocytes, the inventor also described in Japanese Patent Application No. 419158/1990, the development and application of HGF as an epithelial cell growth accelerator for wound healing and skin ulcer treatment or as a medicine for the proliferation of hair root cells. In particular, HGF does not have carcinogenic effect and the activity of promoting the proliferation of cancer cells, which are observed with many growth factors such as EGF, and hence it is more practicable. The inventor, moreover, in Japanese Patent Kokai No. 25010/1994, described that HGF could be used as an anti-cancer agent utilizing the property of HGF to inhibit the proliferation of cancer cells such as HepG2 cell line derived from a human hepatoma, IM9 cell line derived from lymphoblast tumor and the like.

More recently, the inventor discovered that HGF promotes regeneration in injured lung, and that the plasma HGF level in patients with lung diseases is far higher than that in normal subjects (Yanagita et al., Biochem. Biophys. Res. Commun., 182, 802-809, 1992).

Relating to the receptor of the HGF, it has been identified from the recent studies that c-met proto oncogene codes the HGF receptor (Bottaro et al., Science 251, 802-804, 1991; Naldini et al., Oncogene 6, 501-504, 1991).

Another important point in considering the practical use of HGF as medicine is that HGF promotes the growth of cells only in phase G1, that is, the cells only in the growth period, not cells in phase G0, that is, stationary period. It means that it promotes growth and regeneration of injured tissues, but does not act at all on intact tissues. Therefore, if HGF is administered excessively, or if HGF reaches non-ailing sites through blood or the like, it does not induce carcinogenic act ion or excessive growth in normal tissues.

Since HGF widely promotes growth of epithelial cells, as well as hepatocytes, and has the growth inhibitory activity for cancer cells, it is expected that HGF acts to heal tissue injuries in the body. HGF producing cells are not epithelial cells themselves, but it is elucidated that HGF is produced mainly by mesenchymal cells, for example, Kupffer cells and vascular endothelial cells of sinusoidal wall in the liver, capillary endothelial cells in the kidney, alveolar macrophage and vascular endothelial cells in the lung, and it has been elucidated that HGF is supplied from adjacent cells when required, and the so-called paracrine mechanism is established.

However, when the liver or kidney is injured, production of HGF is increased also in intact organs such as the lung, and it is estimated that HGF is supplied also by the so-called endocrine mechanism.

Thus, the HGF is a growth factor acting to heal wounds in various organs and tissues, but in the event of disorder in brain and nerve, it has not been known whether HGF contributes to restoration of brain and nerve injuries or not.

Accordingly, the inventor studied the action of HGF in the brains and nerves, and found that survival of brain and nerve cells is promoted by HGF, and that expression of HGF mRNA and c-met mRNA in the brains is evidently increased in the body having brain injury.

The invention is based on such findings, and it is hence a primary object of the invention to present a useful therapeutic agent for disorder in brain and nerve for prevention and treatment of disorder in brain and nerve.

Disclosure of the Invention

The invention relates to a therapeutic agent for disorder in brain and nerve containing an effective amount of HGF and if necessary, a pharmacologically acceptable carrier.

Other aspects of the invention include a method of treatment of disorder in brain and nerve of humans or mammals by administering an effective amount of HGF; a use of HGF for manufacturing a therapeutic agent for disorder in brain and nerve; an agent promoting survival of brain and nerve cells containing an effective amount of HGF and if necessary, a pharmacologically acceptable carrier; a method of promoting survival of brain and nerve cells of humans or mammals by administering an effective amount of HGF; and a use of HGF for manufacturing an agent promoting survival of brain and nerve cells of humans or mammals.

HGF as described above may be derived from either human or animal tissue or blood components, or manufactured by recombinant DNA technique.

HGF, the active ingredient, possesses an action for promoting survival of brain and nerve cells, and hence can regenerate and restore the injured brains and nerves.

Brief Description of the Drawings

Fig. 1 is a photograph of Northern blot analysis when RNA was electrophoresed in agarose/formaldehyde gel, showing changes of HGF mRNA and c-met mRNA levels in the brain of rats from late-fetal to adult stages.

Fig. 2 is a photograph of Northern blot analysis when RNA was electrophoresed in agarose/formaldehyde gel, showing regional expressions of HGF mRNA and c-met mRNA in adult rat brain.

Fig. 3 is graphs showing effects of HGF and other growth factors on the growth of PC12 cells. In the graphs, A shows the promoting effect of HGF on growth of PC12 cells, B shows the effects of HGF, EGF, NGF, and their combination on growth of PC12 cells.

Fig. 4 is a graph showing the effect of HGF to promote survival of PC12 cells.

Fig. 5 is a photomicrograph showing morphological changes of PC12 cells cultivated in the presence or absence of HGF, EGF or NGF.

Fig. 6 is graphs showing the results of binding experiment of ^{125}I-HGF to PC12 cells cultured in the presence or absence of NGF. In the graphs, A represents the saturation curve of specific binding of ^{125}I-HGF to PC12 cells cultured in the absence of NGF (●) or in the presence of 50 ng/ml of NGF (○), and B represents Scatchard plot of the binding of ^{125}I-HGF to PC12 cells.

Fig. 7 is a photomicrograph showing the morphology of hippocampal nerve cell, showing that HGF prolongs survival of hippocampal nerve cell in primary culture.

Fig. 8 is a photograph of Northern blot analysis when RNA was electrophoresed in agarose/formaldehyde gel, showing induction of HGF mRNA and c-met mRNA expression in brain after experimental cerebral ischemia.

Best Modes of Carrying Out the Invention

As to HGF which is the active ingredient of the invention, any HGF can be used in the invention as long as it is purified to be able to use for a medicine, regardless of preparation methods of HGF. Many methods are known to prepare HGF, and, for example, HGF can be obtained by extraction and purification from organs such as liver, spleen, lung, bone marrow, brain, kidney, placenta and the like, blood cells such as platelets, leukocytes and the like, plasma and serum of mammals such as rat, cow, horse, sheep and the like. Also, it is possible to obtain HGF by cultivation of primary culture cells or cell lines producing HGF, followed by separation and purification from the culture product (e.g. culture supernatant, cultured cell, etc.). Further, HGF can be obtained by gene engineering method which comprises recombining the gene coding HGF with a proper vector, inserting it into a proper host cell to give a transformant, and separating the desired recombinant HGF from the culture supernatant of the transformant (e.g. Nature, 342, 440, 1989, Japanese Patent Kokai No. 111383/1993, Biochem. Biophys. Res. Commun., 163, 967, 1989). The host cell is not specifically limited, and various host cells conventionally used in gene engineering methods can be used, which are, for example, Escherichia coli, Bacillus subtilis, yeast, filamentous fungi, and plant or animal cells.

More specifically, the method of extracting and purifying HGF from live tissues is, for example, to administer carbon tetrachloride to a rat intraperitoneally, remove a liver from the rat with hepatitis, grind it, and purify by the ordinary protein purifying technique such as gel column chromatography using S-Sepharose and heparin Sepharose, HPLC and

the like. Further, by the gene engineering method, the gene coding the amino acid sequence of human HGF is recombined with a vector such as bovine papilloma virus DNA and the like to obtain an expression vector, and by using this expression vector, animals cells such as Chinese hamster ovary (CHO) cells, mouse C127 cells, monkey COS cells and the like are transformed, and HGF can be obtained from the culture supernatant of the transformants.

As to HGF thus obtained, there are possibilities that a part of the amino acid sequence will be deleted or substituted with other amino acid(s), that another amino acid sequence is partially inserted, that 1, 2 or more amino acids are attached to the C and/or N terminals, or that sugars are similarly deleted or substituted. Such HGF analogues are disclosed in Japanese Patent Kokai No. 130091/1992 and PCT International Publication No. WO90/10651, and they may be also used in the invention and are included within the scope of the invention.

HGF, the active ingredient of the therapeutic agent of the invention, possesses the action of promoting survival of brain and nerve cells as shown in Examples below, and it is also known to increase evidently the expression of HGF mRNA and c-met/HGF receptor mRNA in the brain in the body having a injury to brain.

More specifically, HGF mRNA and c-met mRNA were expressed in the brains of late-fetal, neonatal and adult rats. Both HGF mRNA and c-met mRNA were widely expressed in the entire brain of adult rat, and distributions of relatively high level of their expressions were noted in hippocampus, olfactory bulb and their related regions. Similarity of distribution between HGF mRNA and c-met mRNA suggests that HGF plays the role in the brain, same as in the liver, kidney and lung.

Although HGF has been shown to regulate growth, motility and morphogenesis of various types of cells, whether neuronal-type of cells respond to HGF has been unknown. Rat pheochromocytoma PC12 cells are cells derived from neural crest, and have an adrenergic property (Greene et al., Proc. Natl. Acad. Sci., 73, 2424-2428, 1976). The PC12 cells have been widely used in the study of neurotrophic factor-induced differentiation. The cells show many of the properties of adrenal medullary chromaffin cells and by culturing in the presence of NGF, fibroblast growth factor (FGF) or interleukin 6 (IL-6), the cells show a program of physiological changes, resulting in phenotype resembling that of sympathetic nerve cells (Togari et al., Biochem. Biophys. Res. Commun., 114, 1189-1193, 1983).

Although HGF did not stimulate the DNA synthesis of PC12 cells, HGF stimulated viability of PC12 cells, which resulted in prolonged survival. It may be, therefore, concluded that HGF functions as a survival factor for PC12 cells, rather than mitogen. This ability to prolong survival of nerve cells (neurons) is a novel biological activity of HGF.

Same as the result disclosed in literature (Greene et al., Proc. Natl. Acad. Sci., 73, 2424-2428, 1976), NGF inhibited the DNA synthesis and proliferation of PC12 cells while induced their differentiation. Since NGF is known to prevent the death of PC12 cells in serum-free culture medium (Greene, L., J. Cell Biol., 78, 747-755, 1978), NGF maintains the survival of PC12 cells with differentiated phenotype. In contrast to NGF, although HGF had no effect on DNA synthesis of PC12 cells, HGF enhanced cell growth presumably through its remarkable ability to prolong the survival of PC12 cells. Therefore, HGF promotes the survival of PC12 cells through different pathway from NGF and the effect of HGF seems to be similar to that of EGF. However, since the effects on HGF and EGF appeared to be additive in growth promoting activity, these factors seem to regulate growth and survival of PC12 cells through similar but different intracellular signaling pathway.

The presence of high affinity HGF receptor on PC12 cells (185 sites/cell with Kd of 40 pM) clearly indicates that PC12 cells are target cell of HGF and prolonged survival of PC12 cells is mediated through high affinity receptor. On the other hand, induction of differentiation in PC12 cells by NGF accompanied marked decrease in the number of HGF receptor. As known from this result of experiment, HGF may exert its biological activities for the undifferentiated PC12 cells rather than for the differentiated cells. Such differentiation-related decrease in growth factor receptor on PC12 cells was also noted in the report of EGF (Huff et al., Biochem. Biophys. Res. Commun., 89, 178-180, 1979, etc.). Accordingly, it is possible to speculate that alteration of PC12 cells from proliferative to differentiative phase may accompany the unavailability of growth factor receptors and such changes may lead the difference in responsiveness of undifferentiated and differentiated cells to growth factors. T98G, GOTO and SCCH-26 cells derived from central nervous tissues also express high affinity HGF receptor. HGF did not stimulate DNA synthesis of these cells, but these cells may have potential to respond to HGF.

It is a completely novel finding that HGF prolonged survival of hippocampal neurons in primary culture, wherein these cells seem to maintain their original characters of nerve cells (neurons) in vivo. This result suggests that HGF may function as a survival factor for neurons also in vivo. As the phenomenon for supporting this fact, the expression of both HGF mRNA and c-met/HGF receptor mRNA markedly increased after ischemic lesions in adult rat brain.

As stated above, HGF seems to act as a "trophic-factor" for regeneration of various organs and tissues. Taken together with the results, HGF has a possible role as a "trophic-factor" in brain and nerve to prevent degenerative death of neurons and other cells and to prolong survival of nerve cells against various injuries in brain and nerves. The intrinsic biological activities of HGF (cell growth promotion, cell motility promotion, and morphological induction), and activities of HGF as a putative inducer on nervous tissues and as a survival factor on neurons all indicate that HGF is playing an extremely important role in tissue induction of brain and nerves, organogenesis and maintenance of homeostasis of body.

Thus, HGF has the action to prolong survival of brain and nerve cells, and in the body having injury in the brain or nerves, expression of HGF mRNA and c-met/HGF receptor mRNA increases significantly in the brain or nerves. Therefore, the therapeutic agent for disorder in brain and nerve of the invention is useful for nerve degeneration, cerebral stroke, cerebral infarction, dementia, cranial injury, peripheral neuropathy, diabetic neuropathy, neurotoxin induced lesions, injury of nerve cell by surgery, lesions of nerve cell by infection, tumor of nerve cell and the like. Herein, the nerve degeneration may be atrophic or degenerative dropout diseases of nerve cells, including, for example, Alzheimer's disease, senile dementia of Alzheimer type, amyotrophic lateral sclerosis, Parkinson's disease, Huntington's chorea and the like. The peripheral neuropathy includes, for example, lesions in optic nerve, sensory neurons, motor neurons, autonomic neurons and the like.

The therapeutic agent of the invention may be prepared in various preparation forms (for example, liquid, tablet, capsule), and generally it is prepared in the form of injection containing HGF as the active ingredient alone or together with common carrier, or in the form of oral preparation together with common carrier. The injection may be prepared by the conventional method, and for example, HGF is dissolved in a proper solvent (for example, sterilized water, buffer solution, physiological saline), filtered and sterilized, and put in a container aseptically. The content of HGF in the injection may be usually 0.0002 to 0.2 w/v%, preferably 0.001 to 0.1 w/v%. As oral preparation, it is manufactured in various preparation forms, including tablet, granule, fine granule, powder, soft or hard capsule, liquid, emulsion, suspension or syrup, and these preparations may be manufactured by the conventional method. The HGF content in the preparation may be properly adjusted depending on the preparation form and the disease to be treated.

In production of the preparation, it is preferable to add a stabilizer, and examples of the stabilizer include albumin, globulin, gelatin, mannitol, glucose, dextran, ethylene glycol and the like. Moreover, the preparation of the invention may contain other additives necessary for pharmaceutical preparation, such as an excipient, a dissolving aid, an antioxidant, a pain-alleviating agent, an agent for isotonicity and the like. In liquid preparation, it is preferable to store it under frozen conditions or after the removal of water by a process such as freeze-drying. The freeze-dried preparation is used by dissolving again in distilled water for injection and the like before use.

The preparation of the invention is administered through various routes depending on the preparation form. For example, the injection is administered by intrabrain, intravenous, intraarterial, subcutaneous, intramuscular and the like. The dose is adjusted properly depending on symptoms, age and body weight of patient, and generally 0.01 mg to 100 mg of HGF is administered once or several times per day.

Industrial Applicability

In the therapeutic agent and method of treatment according to the invention, HGF which is the active ingredient prolongs survival of brain and nerve cells, and regenerates and restores the injured brain and nerves. Hence, the invention is useful for effectively preventing and treating various disorder in brain and nerve (e.g. dementia, Alzheimer's disease, senile dementia of Alzheimer type, amyotrophic lateral sclerosis, Parkinson's disease, cerebral stroke, cerebral infarction, cranial injury. etc).

Example

The present invention is described in more detail referring to Examples and Preparation Examples. However, the invention is not limited to these examples. Materials and methods used in the following experiments are as follows:

Materials and Methods

(1) Materials

Male Wistar rats were used in the following experiments. Hybond-N, [α-^{32}P]dCTP and Na[^{125}I] and Megaprime DNA labeling system were obtained from Amersham Co. Biodyne-B was from Pall Co. (East Hills, N.Y.). Random primer DNA labeling kits and Oligotex dT30 (trademark) were purchased from Takara Co. (Kyoto) and Roche Pharmaceuticals (Tokyo), respectively.

(2) Growth factors

Human recombinant HGF was purified from the conditioned medium of CHO cells transfected with human recombinant HGF cDNA (Nakamura, et al., Nature 342, 440-443, 1989; Seki, et al., Biochem. Biophys. Res. Commun. 172, 321-327, 1990). 2.5S nerve growth factor (NGF) purified from mouse submaxillary glands was purchased from Biomedical Technology Inc. (Stoughton, MA). Human recombinant epidermal growth factor (EGF) was provided from Earth Pharmaceutical (Akoh, Japan).

(3) Northern hybridization

For Northern analysis, total RNA was purified by the acid guanidinium thiocyanate-phenol-chloroform method. Total RNA was separated by 1.0% agarose/formaldehyde gel electrophoresis and transferred to a Biodyne-B nylon membrane.

EcoR1 fragment (1.4kb) of rat HGF cDNA (RBC-1 clone which encodes the fourth kringle domain of α-chain, the entire β-chain, and a part of 3'-noncoding region), rat c-met cDNA (0.8kb) or rat glutaraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA which were prepared by polymerase-chain-reaction (PCR) amplification was labeled with [α-^{32}P]dCTP using the random primer DNA labeling kits or Megaprime DNA labeling system, and was used as a probe.

Hybridization were performed at 42 °C for 24 h in solution composed of 50% (w/v) formamide, 5 x NaCl/Pi/EDTA (0.18 M NaCl, 10 mM NaH_2PO_4, pH 7.7, 1 mM Na_2EDTA), 2 x Denhardt's, 1.0% SDS, 0.3% sodium N-lauroyl-sarcosinate and 100 µg/ml salmon sperm DNA. The membrane was washed with 0.2 x NaCl/Pi/EDTA - 0.1% SDS for 8 min at 65 °C, then was autoradiographed on Fuji X-ray film, at -70 °C, using intensifying screens.

(4) Cell culture

PC12 rat pheochromocytoma, T98G human glioblastoma, GOTO and SCCH-26 human neuroblastoma cells were obtained from the Japanese Cancer Research Resources Bank. PC12 cells were cultured in RPMI1640 medium supplemented with 12% fetal calf serum (FCS). T98G cells were cultured in Eagle's minimum essential medium (MEM) supplemented with non-essential amino acids (8.9 mg/liter L-alanine, 15.0 mg/liter L-asparagine, 13.3 mg/liter L-aspartic acid, 14.7 mg/liter L-glutamic acid, 11.5 mg/liter L-proline, 10.5 mg/liter L-serine, and 7.5 mg/liter glycine), 1.1 mg/ml pyruvate and 10% FCS. GOTO cells were cultured in mixture of RPMI1640 and MEM medium (1:1) supplemented with 10% FCS, SCCH-26 cells were cultured in ES medium supplemented with 10% FCS.

(5) Radioiodin [^{125}I]-labeling of HGF

Human recombinant HGF was radioiodinated by the chloramine-T method. Details on the methods for the radioiodination were described previously (Higuchi & Nakamura, Biochem. Biophys. Res. Commun. 176, 599-607, 1991). Briefly, 1.5 M sodium phosphate buffer, pH 7.0 (10 µl), 0.5 µg HGF (17 µl), and 0.5 mCi Na[^{125}I] (14Ci/mg I, IMS 30) were mixed in a siliconized tube and the reaction was started by adding 5 µl of chloramine-T solution (100 µg/ml), four times at 30 s intervals. The reaction was stopped by adding 20 µl of 50 mM N-acetyl-L-tyrosine (Sigma Co.), 200 µl of 60 mM potassium iodide and 200 µl of urea solution (1.2 g/ml in 1 M acetic acid). ^{125}I-HGF was separated by molecular sieve chromatography on a Sephadex G-25 column (Pharmacia Co.) equilibrated with 4 mM HCl, 75 mM NaCl, and 1 mg/ml bovine serum albumin (BSA, Sigma Co.). ^{125}I-HGF thus prepared had a specific activity of 70-160 mCi/mg protein.

(6) ^{125}I-HGF binding assay

The binding assay on cultured cells was carried out as follows. PC12, T98G, GOTO, SCCH-26 cells were detached from culture plates by extremely short-term treatment with trypsin. The cell suspension were incubated at 10 °C for 1 h in binding buffer containing various concentrations of ^{125}I-HGF, with or without excess amounts of unlabeled HGF, in siliconized tubes (Assist Co.). The cells were overlaid onto an oil cushion composed of di-n-butyl phthalate and di-(2-ethylhexyl) phthalate (3:2) and centrifuged for 5 min at 12,000 g at 4 °C. After discarding the aqueous and oil phases, ^{125}I-HGF specifically bound to the cell pellet was counted in a γ-counter. All binding experiments were done in triplicate.

(7) Measurement of cell growth, survival, and DNA synthesis of PC12 cells

To measure cell growth, cells were seeded at 10^4 cells/cm^2 on 6-well plates (Corning Co.) pre-coated with collagen and cultured for 24 h. The medium was changed to fresh medium containing 5% FCS and growth factors were added. The medium was changed every third days and growth factors were added in each time. The numbers of the cells were counted using a hematocytometer following dissociation of the cells by trypsin treatment. Data are averaged of triplicate measurements.

To determine survival of PC12 cell, cells were plated at $5x10^4$ cells/cm^2 on 6-well plates and cultured for 24 h. The medium was changed to fresh medium containing 1% FCS.

For measurement of DNA synthesis, PC12 cells were plated at a density of 10^5 cells/well on a collagen coated 24-well plate (Costar Co.). The next day, the medium was changed to fresh medium containing lower concentration of FCS (2.5%) and cultured for 24 h. Growth factors were added and the cells were cultured for 24 h followed by labeling with 1 µCi of ^{125}I-deoxyuridine (2200 Ci/mmol, New England Nuclear) for 12 h. Cultures were washed once with PBS and

once with 10% (w/v) ice cold TCA. Cells were solubilized with 1 M NaOH and radioactivity incorporated into nuclei was counted in a γ-counter.

(8) Protein assay

Protein concentration was measured by micro BCA protein assay system (Pierce Chemical Co.) using bovine serum albumin as the standard.

(9) Primary culture of hippocampal neurons

Hippocampani were dissected from embryonic day 18 rats and incubated for 8 min at 37 °C in 0.25% trypsin. The solution was removed and residual trypsin was inactivated with appropriate amount of FCS or horse serum (HS). The cells were dissociated by trituration through plastic tips. The dissociated neurons were plated into 48-well plates (Costar Co.) precoated with polyethyleneimine (Sigma Co.) at a density of 10^5 cells/cm^2. The neurons were grown in a mixture of Dulbecco's modified Eagle's (DME) medium and Ham's F12 medium (1:1) supplemented with 5% FCS and 5% HS in a 90% air/10% CO_2 humidified incubator (37 °C). The culture medium was replaced with DME containing 10% Nu-Serum (Collaborative Research Co.) in place of the FCS or HS at 12-24 h after seeding.

(10) Experimental cerebral ischemia

9 weeks-old male wistar rats were used for the operation. Cerebral ischemia was induced by insertion of embolus into the right internal carotid artery to stop the blood flow into the middle cerebral artery. The embolus was inserted for 2 h and then blood flow was recirculated. Following appropriate period after recirculation, animals were killed and right and left-brains were removed separately.

Example 1

Changes in HGF mRNA and c-met mRNA levels in rat brain

Changes in HGF mRNA and HGF receptor levels in brain during rat development were examined by Northern blot analysis mentioned before.

Namely, total RNA (50 μg/lane) was electrophoresed in a 1.0% agarose/formaldehyde gel and transferred to a Biodyne-β filter. The membrane was hybridized with a ^{32}P-labeled rat HGF cDNA and rat c-met cDNA probe as described in Materials and Methods. The results are shown in Fig. 1. In Fig. 1, the lower photographs show the signals of the glutaraldehyde 3-phosphate dehydrogenase (GAPDH) as internal controls.

Fig. 1 shows changes in HGF mRNA and c-met mRNA expression in rat brain during late-fetal, neonatal and adult stages. HGF mRNA was detected at very low level in the brain at late-fetal stage, while it increased after birth and reached a maximum at adult stage. On the other hand, c-met mRNA level was expressed in late-fetal brain and it increased markedly after birth, reaching a maximum at day 5. However, c-met mRNA level was remarkably decreased until adult stage. This result that HGF and its receptor mRNAs are continuously expressed in brain from late-fetal to adult stages suggested that HGF may have some roles in brain.

To investigate possible role of HGF in brain, then regional expressions of HGF mRNA and c-met mRNA in brain were examined and effects of HGF on neural cells in vitro were analyzed by the methods mentioned below.

Example 2

Regional expressions of HGF mRNA and c-met mRNA in adult rat brain

Regional expressions of HGF mRNA and c-met mRNA in adult rat brain were examined by Northern blot analysis mentioned before.

Namely, 30 μg or 50 μg per lane of total RNA was electrophoresed, respectively, and transferred to a Biodyne-β filters. The membrane was hybridized with ^{32}P-labeled rat HGF cDNA and rat c-met cDNA probes as described in Materials and Methods. The results are shown in Fig. 2. In Fig. 2, the lowerest photograph shows the signals of the 18S and 28S rRNA, visualized by ethidium bromide staining.

Fig. 2 shows the expression of HGF mRNA and c-met mRNA in various regions of adult rat brain. HGF mRNA was detected in various regions in brain and it was expressed at relatively high level in hippocampus, olfactory bulb, cortex, and cerebellum. The c-met mRNA was also expressed in various regions in brain and was expressed at relatively high level in hippocampus and olfactory bulb.

Example 3

Effects on growth and survival of PC12 cells

To examine the functions of HGF in brain, PC12 cell culture system was used.

① First, to examine the effect of HGF on proliferation of PC12 cells, PC12 cells were cultured in medium containing 5% FCS in the presence or absence of HGF. Namely, PC12 cells were plated on collagen-coated 6-well plates at a density of 10^5 cells/well. The next day, medium was changed to fresh medium containing 5% FCS and the cells were cultured for given days in the absence (○) or presence of 1 ng/ml (●), 3 ng/ml (△) or 10 ng/ml HGF (▲), and the numbers of cells were counted (see Materials and Methods). The results are shown in Fig. 3A. Each value represents mean of triplicate measurements and standard deviations were below 0.3% of each value.

As shown in Fig. 3A, the stimulatory effect of HGF on proliferation of PC12 cells was seen from day 4 to day 10 in culture, and HGF increased the number of the cells in a dose-dependent manner: 1.1, 1.3 and 1.4-fold increase were seen by 1, 3, 10 ng/ml HGF, respectively.

② Since the proliferation of PC12 cells is known to be ceased by NGF, but enhanced by EGF, effects of HGF, EGF, NGF and their combinations on the growth of PC12 cells were compared. PC12 cells were cultured as described in above ①, and growth factors were used as following concentrations: HGF 10 ng/ml; EGF 10 ng/ml; NGF 20 ng/ml. Cell number was determined on 8th day after plating of the cells. The results are shown in Fig. 3B. Each value represents mean of triplicate measurements and standard deviations were below 0.3% of each value.

As shown in Fig. 3B, the number of the cells was increased 1.4-fold by the addition of 10 ng/ml HGF while 2.1-fold increase was seen by the addition of 10 ng/ml EGF. The combination of 10 ng/ml HGF and 10 ng/ml EGF resulted in 2.4-fold increase in cell number compared to that seen in the absence of growth factors. Thus, HGF and EGF additively stimulated the proliferation of PC12 cells. On the other hand, the proliferation of PC12 cells was not affected by 20 ng/ml NGF. Moreover, the number of PC12 cells was not increased by HGF when simultaneously added with NGF.

③ To examine whether HGF induces mitogenesis in PC12 cells, the effect of HGF on DNA synthesis was examined. Namely, PC12 cells were plated at a density of 10^5 cells/well on a collagen-coated 24-well plate. Uptake of ^{125}I-deoxyuridine were measured as described in Materials and Methods. The results are shown in Table 1. Each value represents mean of triplicate measurements and standard deviations.

As shown in Table 1, neither HGF nor EGF did significantly stimulate DNA synthesis of PC12 cells, whereas NGF inhibited DNA synthesis dose-dependently.

Table 1

Growth factor	^{125}I-deoxyuridine uptake (cpm/μg protein)				
	0 ng/ml	1 ng/ml	5 ng/ml	10 ng/ml	20 ng/ml
None	380±1.8	----	----	----	----
HGF	----	366± 6.9	367±4.3	382± 9.7	352± 2.1
EGF	----	395±10.1	380±6.5	387±10.5	369± 8.9
NGF	----	380± 1.8	277±3.1	259± 6.3	218±10.0
5% FCS	492±7.1	----	----	----	----

④ Since HGF did not enhance the proliferation of PC12 cells as mentioned above, the effect of HGF on survival of PC12 cells cultured in the medium containing 1% FCS was examined, in order to examine whether HGF prolongs survival of PC12 cells.

Namely, PC12 cells were plated on collagen coated 6-well plates at a density of $5x10^5$ cells/well. The next day, the medium was changed to fresh medium containing 1% FCS and cultured in the presence of HGF at concentrations of 1 ng/ml (●), 3 ng/ml (△), 10 ng/ml (▲) and in the absence of HGF. The results are shown in Fig. 4. Each value represents mean of triplicate measurement and standard deviations were below 0.6% of each value.

As shown in Fig. 4, in the absence of HGF, about 40% of the total cells died from culture dishes during 4 days of culture period and most of the cells died until day 10 in culture. In contrast to it, the decrease in cell number was not observed in the presence of HGF. The initial number of PC12 cells was maintained at least during 13 days of

culture period in the presence of HGF and 1 ng/ml HGF was fully effective on survival of PC12 cells in this condition.

⑤ Effects of HGF, EGF and NGF on differentiation of PC12 cells were examined. Namely, PC12 cells were cultured in the medium containing 12% FCS in the absence (A) or presence of 10 ng/ml HGF (B), 10 ng/ml EGF (C), 20 ng/ml NGF (D), 10 ng/ml HGF plus 10 ng/ml EGF (E), 10 ng/ml HGF plus 20 ng/ml NGF (F). Cells were cultured for 7 days in each conditions, and morphological changes of cells were observed. The results are shown in Fig. 5.

As shown in Fig. 5, although PC12 cells showed a round-shaped appearance, addition of NGF induced differentiation of PC12 cells into a sympathetic neuronal phenotype as evidenced by neurite outgrowth (Fig. 5A and D). However, there was no morphological change after addition of HGF and HGF-treated cells were indistinguishable from untreated cells (Fig. 5B). Since neither EGF nor combination of HGF and EGF induced neurite outgrowth (Fig. 5C and E), these growth factors do not induce differentiation of PC12 cells. The simultaneous addition of HGF and NGF resulted in induction of differentiated morphology of PC12 cells, suggesting that intracellular signals triggered by NGF largely overcome those triggered by HGF.

⑥ HGF receptor analyses on PC12 cells and other neuronal cell lines were carried out. Namely, binding of ^{125}I-HGF to PC12 cells cultured in the absence or presence of NGF was measured. The binding of ^{125}I-HGF to PC12 cells was determined as described in Materials and Methods. The results are shown in Fig. 6. In Fig. 6, (A) represents saturation curves of specific binding of ^{125}I-HGF to PC12 cells cultured in the absence (●) or presence of 50 ng/ml NGF (○). (B) represents scatchard plot of the binding of ^{125}I-HGF to PC12 cells.

As shown in Fig. 6A, ^{125}I-HGF specifically bound to the undifferentiated PC12 cells cultured in the absence of NGF. Scatchard analysis of the binding indicates that exponentially growing PC12 cells express binding sites of 185 sites/cell with a Kd value of 40 pM (Fig. 6B). When PC12 cells were differentiated into neuronal phenotype during 7 days' culture in the presence of 50 ng/ml NGF, specific binding of ^{125}I-HGF was remarkably reduced (Fig. 6A). Scatchard analysis revealed that these PC12 cells express binding sites of 15 sites/cell with a Kd value of 27 pM (Fig. 6B). Thus, the binding sites of HGF were reduced from 185 sites/cell to 15 sites/cell during the differentiation of PC12 cells.

Binding of ^{125}I-HGF to other cell lines derived from central nervous system was also measured. The results are shown in Table 2. As shown in Table 2, high affinity HGF receptor was also found in human glioblastoma T98G, human neuroblastoma GOTO and SCCH-26, which express binding sites of 540, 120, 60 sites/cell, respectively, with Kd values of 30-40 pM.

Table 2

Cell	Origin	Kd (pM)	B max (sites/cell)
T98G	Human, glioblastoma	31	540
PC12	Rat, pheochromocytoma	40	185
GOTO	Human, neuroblastoma	30	120
SCCH-26	Human, neuroblastoma	20	60

Example 4

Effect on hippocampal neurons in primary culture

Since it turned out that HGF acts as a survival factor for PC12 cells, tests determining whether HGF has ability to prolong survival of nerve cells in primary cultur were examined. Hippocampal neurons were primarily cultured in the presence or absence of HGF, and morphological appearances of the cells at day 1 and day 6 in culture were measured. The results are shown in Fig. 7. As shown in Fig. 7, when hippocampal neurons cultured in the absence of HGF for 6 days, most of the cells died. The addition of HGF to these cultures resulted in increased number of survived neurons. Thus HGF acts as a survival factor for hippocampal neurons in primary culture.

Example 5

Induction of HGF mRNA and c-met mRNA in brain after cerebral ischemia

Since it is clarified that HGF acts as a survival factor for neurons in primary culture, the protective effect of HGF on neuronal degeneration following cerebral injuries was examined in the experimental cerebral ischemia. The experimental cerebral ischemia was carried out as described in Materials and Methods. Total RNA was extracted from right and left-brain at 4, 8, 12 and 24 h after recirculation, and HGF mRNA and c-met mRNA levels were measured by Northern blot analysis as described in Materials and Methods. The results are shown in Fig. 8. In Fig. 8, the lower photographs show the signals of the 18S and 28S rRNA, visualized by ethidium bromide staining.

In this experimental condition, major ischemic lesions were induced in right-brain, while left-brain was also injured after blood recirculation slightly in retard of right-brain. In right-brain, HGF mRNA was induced from 12 h after recirculation and remarkable induction was seen after 24 h. In left-brain, HGF mRNA increased markedly 24 h after recirculation.

On the other hand, c-met mRNA was markedly induced in the similar time-dependency with that of HGF mRNA. In right-brain, c-met mRNA increased from 12 h after recirculation and remarkable increase was seen 24 h after ischemic treatment. In left-brain, c-met mRNA was induced 12 h after treatment and was markedly increased after 24 h.

On the other hand, only a little induction of both HGF and c-met mRNAs was observed in sham-operated animals.

Preparation Example 1

A solution containing 1 mg of HGF, 1 g of mannitol and 10 mg of polysorbate 80 in 100 ml of physiological saline was aseptically prepared. 1 ml of the solution was poured into each vial and lyophilized, and then the vial was sealed to obtain a freeze-dried preparation.

Preparation Example 2

A solution containing 1 mg of HGF and 100 mg of human serum albumin in 100 ml of 0.02M phosphate buffer (containing 0.15M of NaCl and 0.01% of polysorbate 80, pH 7.4) was aseptically prepared. 1 ml of the solution was poured into each vial and lyophilized, and then the vial was sealed to obtain a freeze-dried preparation.

Preparation Example 3

A solution containing 1 mg of HGF, 2 g of sorbitol, 2 g of glycine and 10 mg of polysorbate 80 in 100 ml of distilled water for injection was aseptically prepared. 1 ml of the solution was poured into each vial and lyophilized, and then the vial was sealed to obtain a freeze-dried preparation.

Claims

1. A therapeutic agent for disorder in brain and nerve, which contains an effective amount of HGF (hepatocyte growth factor) and if necessary, a pharmacologically acceptable carrier.

2. A therapeutic agent for disorder in brain and nerve according to claim 1, wherein HGF is manufactured by gene recombination.

3. A therapeutic agent for disorder in brain and nerve according to claim 2, wherein the host cell for gene recombination is Escherichia coli, Bacillus subtilis, yeast, filamentous fungi, plant or animal cells.

4. A therapeutic agent for disorder in brain and nerve according to claim 1, 2 or 3, wherein the disorder in brain and nerve is nerve degeneration, cerebral stroke, cerebral infarction, dementia, cranial injury or peripheral neuropathy.

5. A method of treatment of disorder in brain and nerve of humans or mammals, which comprises administration of an effective amount of HGF.

6. A method of treatment of disorder in brain and nerve according to claim 5, wherein the HGF is manufactured by gene recombination.

7. A method of treatment of disorder in brain and nerve according to claim 5 or 6, wherein the disorder in brain and nerve is nerve degeneration, cerebral stroke, cerebral infarction, dementia, cranial injury or peripheral neuropathy.

8. A use of HGF for manufacturing a therapeutic agent for disorder in brain and nerve.

9. A use of HGF according to claim 8, wherein the HGF is manufactured by gene recombination.

10. A use of HGF according to claim 8 or 9, wherein the disorder in brain and nerve is nerve degeneration, cerebral stroke, cerebral infarction, dementia, cranial injury or peripheral neuropathy.

11. An agent promoting survival of brain and nerve cells, which contains an effective amount of HGF and if necessary, a pharmacologically acceptable carrier.

12. A method of promoting survival of brain and nerve cells of humans or mammals, which comprises administration of an effective amount of HGF.

13. A use of HGF for manufacturing an agent promoting survival of brain and nerve cells of humans or mammals.

Fig. 1

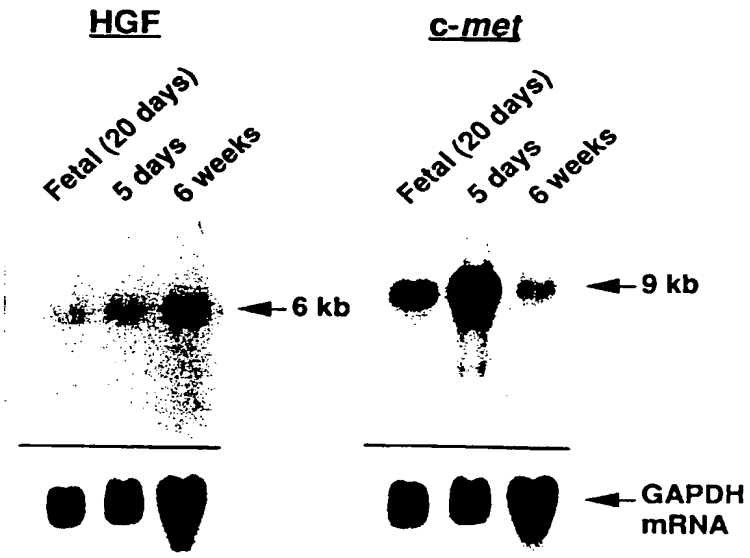

HGF
c-met
Fetal (20 days)
5 days
6 weeks
Fetal (20 days)
5 days
6 weeks
6 kb
9 kb
GAPDH
mRNA

Fig. 2

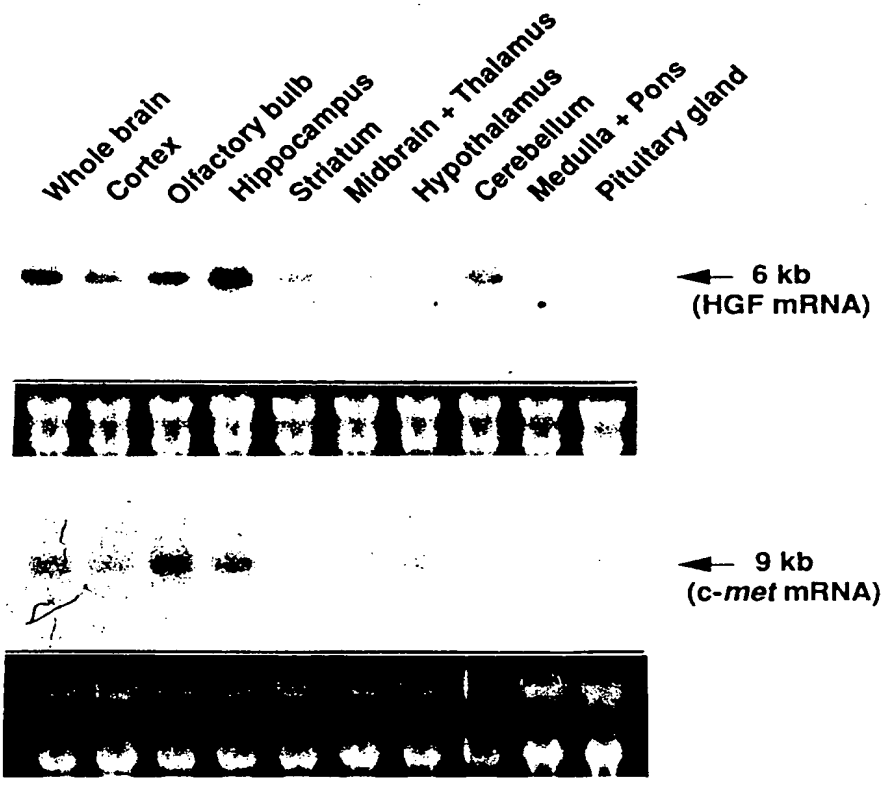

Whole brain
Cortex
Olfactory bulb
Hippocampus
Striatum
Midbrain + Thalamus
Hypothalamus
Cerebellum
Medulla + Pons
Pituitary gland
6 kb
(HGF mRNA)
9 kb
(c-met mRNA)

Fig - 3

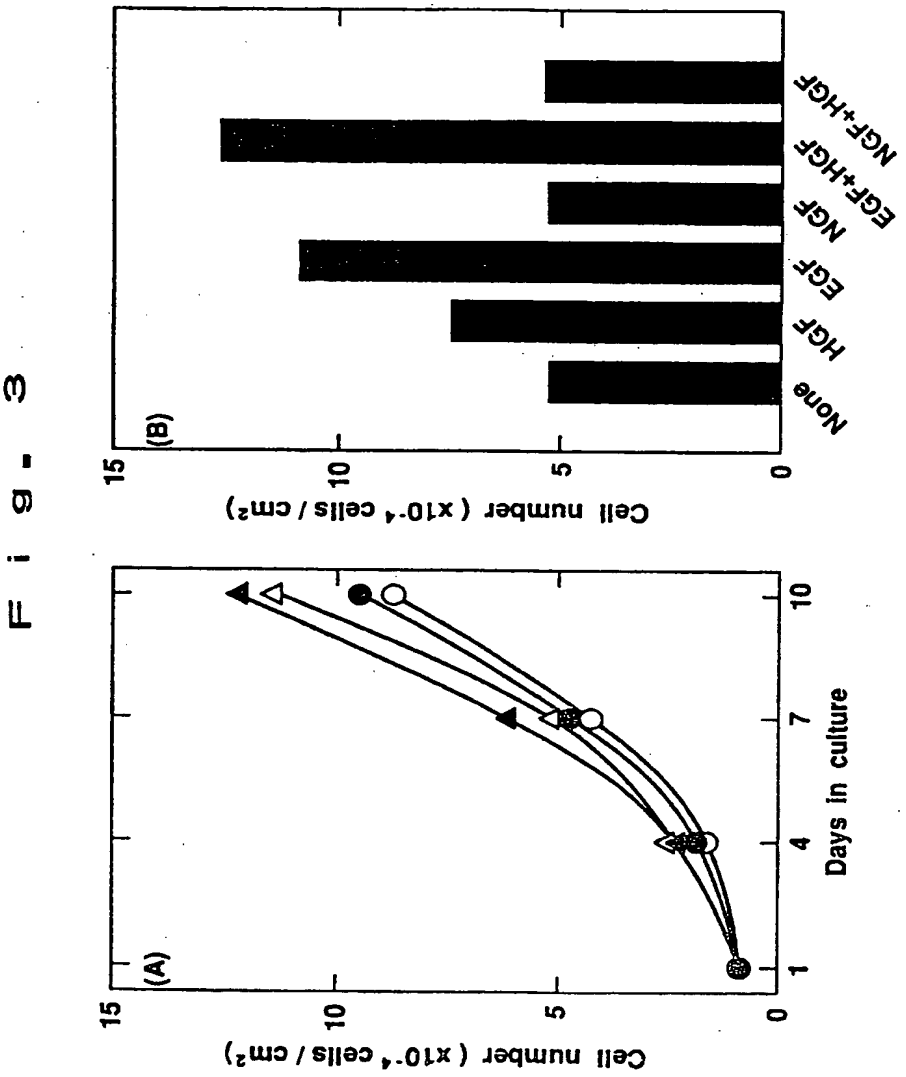

(B)
Cell number (x10⁻⁴ cells / cm²)
0
5
10
15
None
HGF
EGF
NGF
EGF+HGF
NGF+HGF
(A)
Cell number (x10⁻⁴ cells / cm²)
0
5
10
15
1
4
7
10
Days in culture

Fig. 4

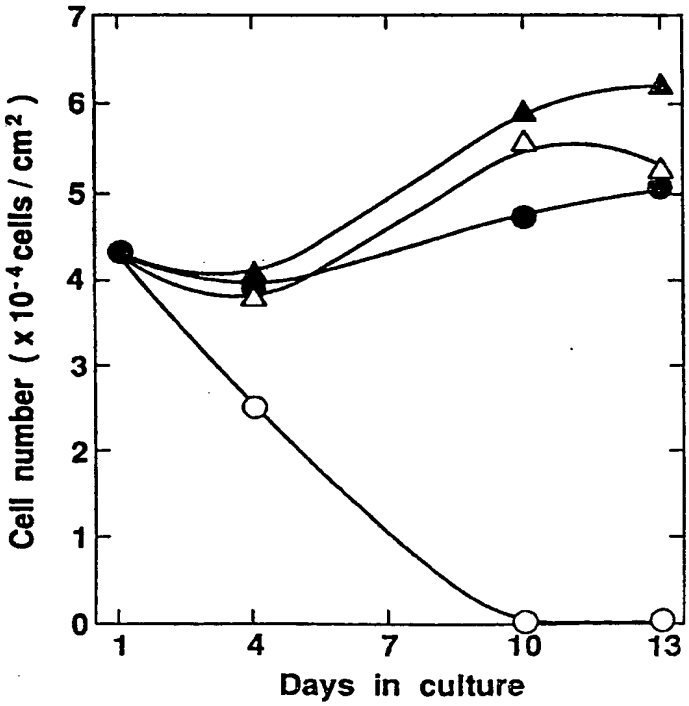

Cell number (x 10⁻⁴ cells / cm²)
0
1
2
3
4
5
6
7
1
4
7
10
13
Days in culture

Fig. 5

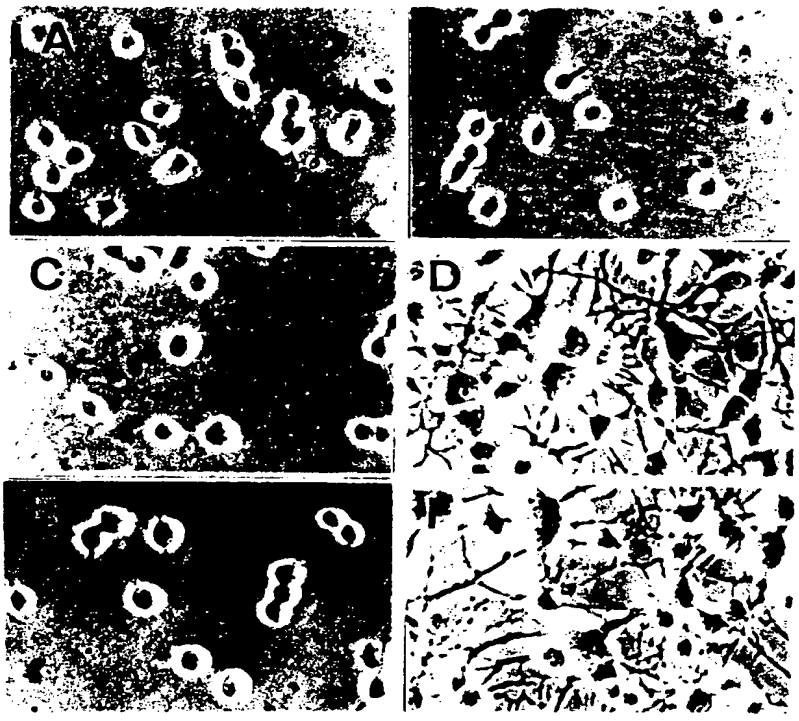


Fig. 6

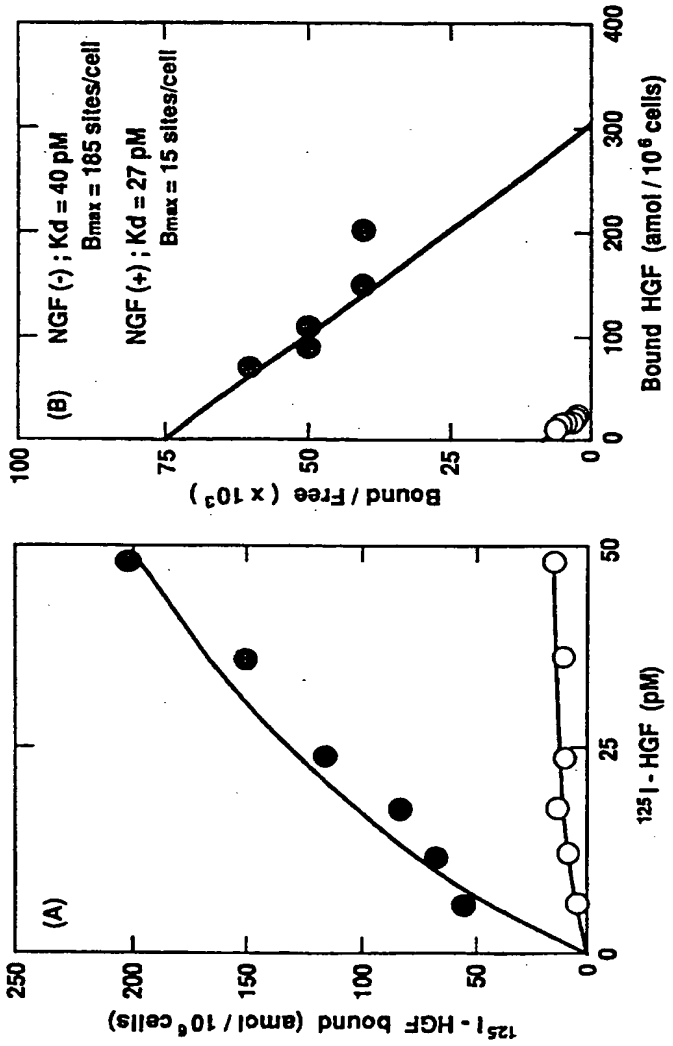

(A)
^{125}I - HGF bound (amol / 10^6 cells)
^{125}I - HGF (pM)
(B)
NGF (-) ; Kd = 40 pM
Bmax = 185 sites/cell
NGF (+) ; Kd = 27 pM
Bmax = 15 sites/cell
Bound / Free (x 10^3)
Bound HGF (amol / 10^6 cells)

Fig. 7

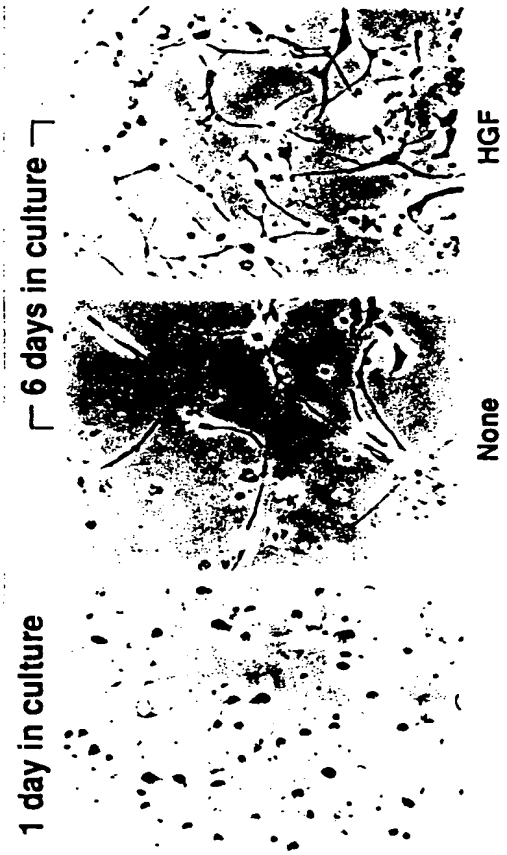
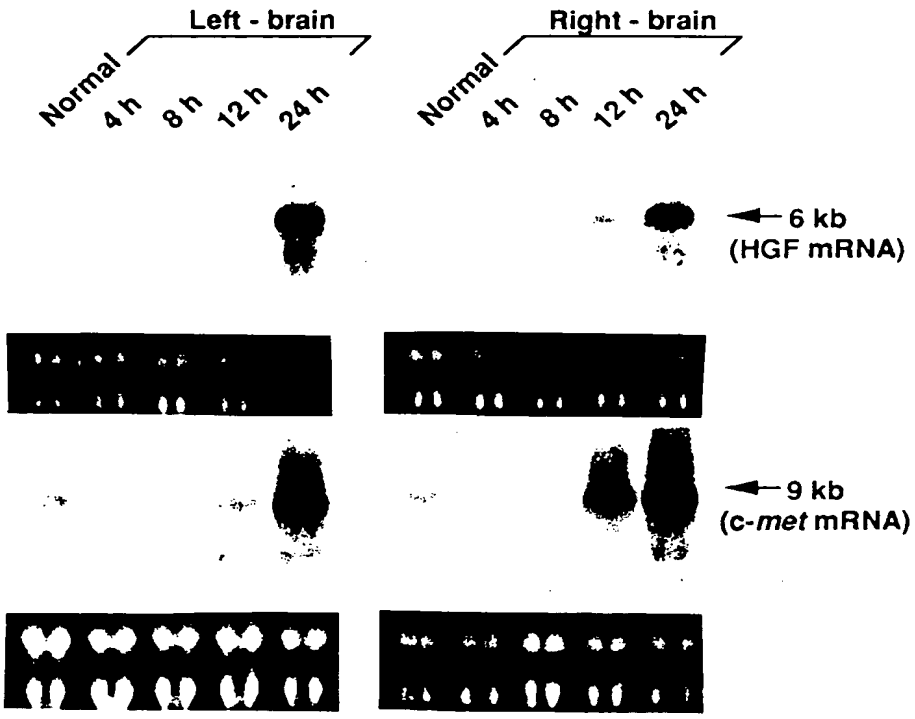


Fig. 8


Left - brain
Right - brain
Normal
4 h
8 h
12 h
24 h
Normal
4 h
8 h
12 h
24 h
6 kb
(HGF mRNA)
9 kb
(c-met mRNA)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP94/01533

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl^6 A61K38/18//C07K14/475

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl^5 A61K37/24, 37/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, A, 5-213733 (Sansho Seiyaku K.K.), August 24, 1993 (24. 08. 93), (Family: none)	1-4, 8-11, 13
A	JP, A, 4-49246 (Toshikazu Nakamura), February 18, 1992 (18. 02. 92) & EP, A, 462549	1-4, 8-11, 13
A	JP, A, 4-18028 (Toshikazu Nakamura and another), January 22, 1992 (22. 01. 92) & EP, A, 456188	1-4, 8-11, 13
A	JP, A, 5-25056 (Max-Plank Institut för Saikaiatory), February 2, 1993 (02. 02. 93) & WO, A, 91/2067 & EP, A, 484416	1-4, 8-11, 13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
November 22, 1994 (22. 11. 94)	December 13, 1994 (13. 12. 94)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)